Expert opinion

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Regarding the evidentiary question: **"What is the power of the RT-qPCR assay and currently used rapid tests to detect SARS-CoV-2 coronavirus infection?"**

1. Nucleic acid detection by RT-qPCR test

Prefix: Reverse **t**ranscriptase-**q**uantitative **p**olymerase **c**hain **r**eaction (RT-qPCR) assays are unsuitable as a diagnostic tool for the detection of an active infection with SARS-CoV-2 for numerous reasons.

1.1 Explanation of terms/basics

In a **polymerase chain reaction (PCR)**, a defined short piece of deoxyribonucleic acid (DNA) (usually 100-1000 bases) is amplified using the enzyme polymerase. The piece of DNA to be amplified is flanced with the help of two very short single-stranded DNA segments, the "primers".

These **primers** usually consist of a defined sequence of 18-25 nucleic acid bases (the primer sequence) that specifically match the regions on the DNA that flank the section to be amplified. To ensure PCR specificity, these primers must explicitly match only this flanking region and no other region of a DNA, neither in the target organism nor in another DNA. With the help of large gene databases and corresponding software programs (e.g. primer blast https://www.ncbi.nlm.nih.gov/tools/primer-blast/), these primers can be selected highly specifically in the PCR design. Specialized companies then synthesize the molecular chains from the submitted primer sequences and deliver them to the PCR laboratory or the manufacturer of PCR kits. Here, these primers must then be tested with valid positive and negative controls under a wide variety of experimental conditions and optimized in use. This ensures that only the DNA searched for is detected and amplified with the primer pair used, and that no other similar DNA segments are detected, a mandatory prerequisite for specificity.

Once the primers have been found and are specific, the DNA to be amplified can be mixed with the primer pair, various auxiliary chemicals and the polymerase enzyme in a reaction batch and the chain reaction started.

PCR procedure: The following steps are repeated in cycles.

1. the mixture is boiled at over 90°C (denatured). This separates the DNA strands, which are usually present as a double strand, into single strands to enable the subsequent attachment of the primers.
2. During the subsequent cooling down to the so-called "**annealing temperature**", the primers can attach to their matching regions on the separated DNA strands. The

binding of the primers, the annealing, only occurs in a narrowly limited temperature range, the so-called melting temperature. This depends mainly on the base composition of the primers and therefore their sequence will ideally always be chosen so that both primers have the same melting temperature of about 60°C. The annealed primers form the DNA strands. The attached primers form the starting point for the polymerase.

3. starting from the primers, this polymerase completes the single-stranded DNA, which is present due to the heating, into a matching double strand (**elongation**), usually at approx. 72°C.

Due to the position of the two primers on the flanking sides of the sought DNA section, the elongation reactions on the single strands are in opposite directions, since the polymerase always works in one direction only. At the end of this step, two identical new double-stranded DNA molecules have now been created from an original double-stranded DNA, which are separated again by boiling, then amplified into 4 identical DNA molecules with the aid of primer addition and the polymerase, and so on.

Each PCR cycle consisting of boiling-annealing-elongation causes a doubling of the DNA section sought, so that the amplification takes place in the logarithm of 2 and thus an extremely high number of copies of the original starting material is available very quickly.

Thus, after 10 PCR cycles, 210 = 1,024 DNA copies are obtained from one DNA strand, after 20 cycles already more than 1 million (1,048,576) and after 30 cycles more than 1 billion (1,073,741,824) copies

In the **quantitative PCR (qPCR)** technique, as currently used worldwide mainly for the detection of genomic RNA from SARS-CoV-2, a third short piece of DNA, similar to the two primers, is used which can bind appropriately in the middle of the DNA section sought, the "**probe**". Unlike the two primers (which are nucleic acids only), this probe is additionally bound to two molecules, a fluorescent dye at one end and another molecule (quencher), which can prevent the emission of fluorescence as long as both are simultaneously (i.e. in close proximity to each other) on the sample. During the elongation step, the polymerase now degrades this probe. This separates the quencher from the fluorescence molecule and the latter can now emit its color signal. This color signal is detected and measured in the device performing the PCR (thermocycler). Thus, with each PCR cycle, more and more fluorescence signals are released according to the increasing number of copies, the probe "glows" more and more. And the curve of color signal intensity increases with each cycle. At a certain value, the curve then exceeds the background noise (threshold) and is considered positive. The number of cycles at which this threshold is exceeded is referred to as the **CT value** (CT stands for "cycle threshold").

The faster the fluorescence rises (lower CT), the more initial copies of the DNA sought were present in the PCR approach. Since neither the primers nor the enzyme polymerase always work 100% specifically, a fraction of non-specific DNA is also copied in each PCR run. And the more cycles the PCR runs, the greater the risk that even these few non-specific reactions will exceed the threshold value. Therefore, from a CT value of 40, a false positive signal due to non-specific starting materials must be assumed with the greatest probability. A reliable PCR should therefore require no more than 30-35 cycles to generate a clear "positive" signal; in the case of active infections with sought-after viruses, a sufficient number of cycles of 25-30 can be assumed (see also point 3.2.).

The **reverse transcriptase reaction (RT)** is required if the starting nucleic acid to be amplified is not present as DNA but as **ribonucleic acid (RNA),** as is the case with SARS-CoV-2 as an RNA virus. Since only DNA can be amplified in PCR, an RNA must first be converted into DNA. This is performed with the help of the enzyme "reverse transcriptase", which creates a complementary copying strand of DNA from RNA, which then serves as the starting material for the PCR.

In order to evaluate the reliability of a result obtained by RT-qPCR or even PCR, the sensitivity and specificity of the test system used are evaluated using defined samples of diluted correct target genes (e.g. RNA of the sought virus, the positive control) and very similar, but not sought target genes (e.g. closely related viruses, negative control to exclude cross-reaction). Further, due to very high amplification cycles, false positives can result from amplification of nonsense signals as well

The **sensitivity** indicates how sensitively the test can detect even the smallest amounts of the target gene sought, while the **specificity** describes how reliably the test excludes the possibility that other, closely related genes can also lead to a positive result (**false positive**). The higher the specificity, the more certain it is that the PCR system itself will not produce false positive results.

However, this does not exclude false positive events, which can be caused by laboratory contamination with target genes, contamination of test chemicals and contamination directly during sample collection. These contamination-related false positive results can be excluded by rigorous quality assurance and standard operating procedures (SOPs), the use of specially trained personnel and permanent external control in the form of interlaboratory comparisons.

1.2 Basic information on diagnostic significance

The inventor of the PCR test, Nobel Prize winner Kary Mullis, who died in August 2019, repeatedly pointed out that his test is solely suitable for making a molecule (deoxyribonucleic acid, DNA) or fragment of DNA, which is otherwise invisible to the human eye, visible by amplification. But not to allow a statement on whether what has been made visible is dangerous or causes illness.

In particular, a PCR test - even if performed correctly - cannot provide any information on whether a person is infected with an active pathogen or not. This is technically impossible, because the test procedure includes a complete destruction of the biological material and separation of nucleic acids from all other material, such destroying any structure necessary for biological function like replication and infection. Further the test cannot distinguish between "dead" matter*, such as a completely harmless genome fragment as a remnant of the body's own immune system's fight against a cold or flu (such genome fragments can still be found many months after the immune system has "taken care" of the problem), and "living" matter, i.e. a "fresh" virus capable of reproducing.

Explicitly, this is listed as a disadvantage of PCR on the information sheet of the Swiss Federal Office for Civil Protection FOCP Spiez Laboratory as follows: "*Only pathogens whose gene sequence is known can be detected*.

Whether a pathogen is infectious (virulent, "alive") or not remains unknown" (https://www.labor-spiez.ch//pdf/de/dok/pos/88_021_Plakate_PCR_d.pdf). Also Marion Koopmanns, the director of the Department of Viral Sciences at Erasmus University and expert advisor to WHO, thus one of the central virologists of the Corona question and at the same time co-author on the RT-qPCR publication of Corman/Drosten in Eurosurveillance confirms in an interview with NPO Radio 1 (26.11. 2020 as part of her podcast "Virusfeiten" https://www.nporadio1.nl/podcasts/virusfeiten/46542/4-blijvend-moe-na-corona-misschien-helpt-een-aspirientje) in response to the presenter's statement that the PCR test does not necessarily show that one is contagious, that this is correct, because the PCR shows whether one has viral RNA on one's person (at minute 0:09 in

https://www.youtube.com/watch?v=flsF7trvq2c)

Explicitly the decisive passage of the interview with Marion Koopmanns (MK):

"MK: ...there are some stories circulating that say, well, the PCR test is not good.

Interviewer: At least it doesn't necessarily show that you're contagious.

MK: Yeah, exactly. And that's also true. Because the PCR shows that you have the viral RNA with you. That's literally what PCR does. And whether that RNA is in a virus particle that is still intact and also infectious. Or whether it's just residual RNA that can be detected long after infection. There is no way to distinguish between the two. You can get a feel for it by looking up "How much is there?". But you can't tell that difference very well. That is: this test is great for saying "you've had it", but this test is less good for saying "at this point you're still infectious".

Interviewer: you're talking about the PCR test right now, aren't you?

MK: Yes."

In original: (Dutch)

" MK: ...er ciruleren wat verhalen waarin gezegd wordt, nou ja, de PCR test ist niet goed. Interviewer: Althans die toont niet persee aan dat je besmettelijk bent.

MK: Ja precies. En dat klopt ook. Want de PCR toont aan dat jij het virus RNA bij je hebt. Dat is letterlijk wat de PCR doet. En of dat RNA in een virus deeltje zit dat nog intact is en ook besmettelijk is. Of dat het gewoon restjes RNA zijn, die je nog een tijd lang nadat iemand geinfecteerd is geweest, kunt aantonen, dat onderscheid zie je niet. Je kunt een beetje een gevoel krijgen door te kijken "hoeveel is het?". Maar dat verschil is niet goed te maken. Dat betekent, die test is prima om te zeggen "je hebt het gehad", maar die test is minder geschikt om te zeggen "op dit moment ben je nog besmettelijk".

Interviewer: Over de PCR test heb je het nu, huh?

MK: ja“

* For example, PCR is also used in forensics to amplify residual DNA present from hair remains or other trace materials by means of PCR in such a way that the genetic origin of the perpetrator(s) can be identified ("genetic fingerprint").

The Swedish Ministry of Health states on its official website (https://www.folkhalsomyndigheten.se/publicerat-material/publikationsarkiv/v/vagledning-om-kriterier-for-bedomning-av-smittfrihet-vid-covid-19/): “*The PCR technology used in tests to detect viruses cannot distinguish between viruses capable of infecting cells and viruses that have been rendered harmless by the immune system**, and therefore these tests cannot be used to determine whether someone is infectious or not.** RNA from viruses can often be detected for weeks (sometimes months) after infection but does not mean that a person is still infectious*.” In the original note: “*PCR-tekniken som används i test för att påvisa virus kan inte skilja på virus med förmåga att infektera celler och virus som oskadliggjorts av immunförsvaret och därför kan man inte använda dessa test för att avgöra om någon är smittsam eller inte. RNA från virus kan ofta påvisas i veckor (ibland månader) efter insjuknandet men innebär inte att man fortfarande är smittsam.*“ This assessment was confirmed on 19.04.2021.

So, even if everything is done "correctly" when performing the PCR including all preparatory steps (PCR design and establishment, sample collection, preparation and PCR performance) and the test is positive, i.e.: detects a genome sequence, which may also exist in one or even the specific "Corona" virus (SARS-CoV-2), this does not mean under any circumstances that the person, who was tested positive, must be infected with a replicating SARS-CoV-2 and thus infectious = dangerous for other persons.

Rather, for the detection of an active infection with SARS-CoV-2, further, and specifically diagnostic methods such as the isolation of reproducible viruses must be used (gold standard).

1.3 Factors influencing the reliability of the PCR test

In fact, however, the results of a PCR test depend on a number of parameters which, on the one hand, cause considerable uncertainties and, on the other hand, can be specifically manipulated in such a way that many or few (apparently) positive results are obtained.

1.3.1 Number of independent target genes ("targets")

The protocol "Diagnostic detection of Wuhan coronavirus 2019 by real-time PCR" (https://www.who.int/docs/default-source/coronaviruse/wuhan-virus-assay-v1991527e5122341d99287a1b17c111902.pdf), originally published by WHO on 13/01/2020, described the sequence of PCR detections of **three independent partial genes** of the virus later renamed SARS-CoV-2. The sequence referred to the E gene, the RdRp gene, and then the N gene. Already on 17.01.2020 a change followed by the WHO with the protocol "Diagnostic detection of 2019-nCoV by real time PCR" (https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf?sfvrsn=a9ef618c_2) in which the N-gene was removed as detection and thus instead of the original three targets only **two targets** were recommended. On March 02, 2020, a WHO test protocol "Laboratory testing for coronaviurs disease 2019 (COVID-19) in suspected human cases" (https://apps.who.int/iris/bitstream/handle/10665/331329/WHO-COVID-19-laboratory-2020.4-eng.pdf?sequence=1&isAllowed=y), which was updated again, pointed out that „.... *In areas where COVID-19 virus is widely spread a simpler algorithm might be adopted in which for example screening by RT-PCR of* ***a single discriminatory target*** *is considered sufficient.....*" (page 3 below), whereupon the laboratories widely switched to analyzing only one target. As a result, many laboratories specialized only in the E gene detection as the sole target as a valid PCR, as e.g. explicitly described by the Augsburg laboratory on 03.04. (only still available in the Internet cache: https://www.oder-spree-piraten.de/wp-content/uploads/2020/05/Ge%C3%A4ndertes-Befundlayout-der-SARS-CoV2-PCR-Ergebnisse-_-Labor-Augsburg-MVZ-GmbH.pdf

However, the outstanding importance of the number of independent target genes analyzed by PCR results from the following calculation:

The three targets E, RdRp and N gene originally specified in the WHO protocol for the detection of SARS-CoV-2 were rapidly used in many laboratory and commercial test systems. An interlaboratory comparison by Institut Instant e.V. (https://corona-ausschuss.de/wp-content/uploads/2020/07/Instand-Ringversuch-Virusgenom-Nachweis-SARS-CoV-2.pdf) showed a mean specificity for these genes of:

Target of the SARS-CoV-2 genome	Number of test analyzed	Specificity A (test sample cell culture without virus)	Specificity B Test Sample contains related Coronavirus (HCoV 229E)	Mean % detected correct	Mean specificity absolute	Mean error rate (1-abs. Spec.)
E-gen	24	99,46%	95,17%	97,31	0,9731	0,0269
RdRp-gen	13	97,80%	90,66 %	94,23	0,9423	0,0577
N-gen	21	98,20%	87,95 %	93,08	0,9308	0,0692

In a mixed population of 100,000 tests, even no true infected person would result due to the mean error rate:

For E-only genetic test: 100,000 x 0.0269 = 2690 false positives.

For E and RdRp test in sequence: 100,000 x (0.0269 x 0.0577) = 155 false positive

For all three genes (E, RdRp, N): 100,000 x (0.0269 x 0.0577 x 0.0692) = 10 false positives

This means that the specification of the WHO to successively reduce the number of target genes of SARS-CoV-2 to be tested from three to one resulted in an increase in the number of persons tested falsely positive in the above calculation example from 10 with three genes to almost 3000 with only the E gene per 100,000 tests performed. If the 100,000 tests carried out were representative of 100,000 citizens of a city/county within 7 days, this question of the target genes used alone would result in a difference of 10 compared to 155 compared to 2690 with regard to the "/-daily incidence" and, depending on this, the severity of the restrictions on the freedom of the citizens taken.

Evaluation: The calculation example also shows how daily case numbers can be manipulated by "playing to the specifications" regarding the targets to be detected for the laboratories. In view of the immense impact on political decisions, which are determined by the absolute numbers of positive tests and the "7-day incidence" derived from them, the specification of the WHO (and also of the RKI) to reduce the target genes has clearly been suitable to artificially inflate the "pandemic" by a factor of 300 by wrong test specifications.

This is an evidence-free procedure, which on the one hand entails enormous personal restrictions of quarantine/isolation, which the falsely "positively tested" persons have to suffer, and on the other hand willingly accepts the enormous social and economic restrictions and damages via the "7-day incidence number".

If the correct target number of three or even better (as e.g. in Thailand) up to six genes had been consistently used for PCR analysis, the rate of positive tests and thus the "7-day incidence" would have been reduced almost completely to zero.

1.3.2 Number of cycles performed (CT value)

In addition to the number of target genes detected, especially in the case of only one or a maximum of two genes, the number of cycles of amplification in the qPCR up to the "positive" result and the resulting CT value are decisive factors. **The smaller the CT value of a sample in a qPCR, the higher the initial amount of DNA in the sample.** Under standardized conditions, this correlates with (in the case of viruses) the initial amount of viruses, the so-called **viral load,** which should ideally be expressed as "number of viral copies" per ml of sample. This viral load also correlates in the case of SARS-CoV-2 with the cultivability of infectious viruses in cell culture as published with the participation of C. Drosten already in March 2020. (Figure 1e in Wölfel et al., https://doi.org/10.1038/s41586-020-2196-x)

Here, a minimum quantity of 10^6 RNA copies/ml was necessary in order to be able to grow viruses from the sample, in another work from the group of C. Drosten (from May 2021) even an average of 10^8 viruses in the sample was necessary for a positive cell culture (supplemental Figure S4 from https://pubmed.ncbi.nlm.nih.gov/34035154/). In the latter work, it was also found that none of the 25,381 individuals tested had viral genomes per ml in the sample in the case of a determined viral load below 10^5 (Table S1), whereas RT-qPCR from the original protocol (Corman V et al., 10.2807/1560-7917.ES.2020.25.3.2000045) can already deliver a positive result at approx. 4 copies per sample preparation (5µl corresponding to approx. 10^3 copies/ml), i.e. already by a factor of 1000-10000 earlier than in a sample with an actual infectious virus load.

Even **commercial PCR test systems**, so-called “kits”, sometimes show detection limits of less than 10 copies/reaction, such as kits from the company TIB-Molbiol (https://www.roche-as.es/lm_pdf/MDx_53-0777_96_Wuhan-R-gene_V200204_09155376001%20%282%29.pdf).

In technical terms, a distinction must be made here between "colonization" of the throat with a few individual viruses that do not cause infection and a genuine "infection". The latter is accompanied by viruses capable of multiplying, which then leads to a) a symptomatic illness and b) infectivity, i.e. the ability to infect other persons.

Christian Drosten already described this aspect in 2014 in an interview in the “Wirtschaftswoche” (https://www.wiwo.de/technologie/forschung/virologe-drosten-im-gespraech-2014-die-who-kann-nur-empfehlungen-aussprechen/9903228-2.html) in connection with MERS: "*Yes, but the method* (note: PCR is meant) *is so sensitive that it can detect a single hereditary molecule of this virus. If such a pathogen, for example, flits across a nurse's nasal mucosa for just one day* (note: this would be the above-mentioned "colonization") *without her becoming ill or otherwise noticing anything about it, then suddenly she is a Mers case. Where previously deathly ill people were reported, now suddenly mild cases and people who are actually perfectly healthy are included in the reporting statistics.*" [....] "*Because what is initially of interest are the real cases* (Note: These are the "infected"). ***Whether asymptomatic or mildly infected hospital workers are really virus carriers, I think, is questionable. Even more questionable is whether they can pass the virus on to others***." The latter is a crucial statement also with respect to the SARS-CoV-2 viruses, which are very closely related to MERS. But it is precisely this point about virus transmission (and thus driving the pandemic) that is the rationale for the intervening measures such as quarantine/isolation orders, the "lockdowns," and the so-called AHA rules.

Further evidence for the relevance of the CT value

A **Canadian study** by Jared Bullard/Guillaume Poliquin in Clinical Infectious Deseases 2020, which can be read at the link (https://doi.org/10.1093/cid/ciaa638), came to the conclusion as early as May 2020, that **no reproducible virus was found above a CT value of 24.** This means that the attempt to subsequently cultivate reproducible viruses from smear samples that only resulted in a positive test at a higher CT value failed. According to this study, above a CT value of 24, the amount of detectable viral genetic material is so low that the positive test could no longer be interpreted in terms of an active infection. A large study by Jaffar et al. (10.1093/cid/ciaa1491) set the limit for the cultivability of SARS-CoV-2 from patient sample material at a CT value of 30.

In a study comparing antigen testing/RT-qPCR and virus cultivation from the CDC (https://academic.oup.com/cid/advance-article/doi/10.1093/cid/ciab303/6224406) , successful virus cultivation was described for a CT range of 17.4-28.8, where only at a CT of <25 all specimens were from symptomatic individuals and were associated with successful virus cultivation. When the CT was between 25 and 29, in 18.2% Virus isolation was positive. In the original: *"Virus was isolated from specimens with Ct values ranging from 17.4-29.8; virus was isolated from all specimens with a Ct value <25 and from 18.5% (5/27) of specimens with a Ct value ≥25.*" (center of page 9). Irrespective of this check using virus cultivation, however, all samples that were positive in two target sequences from the "N gene" with a CT up to 40 were considered "true positive".

In his NDR podcast of Feb. 16, 2021, C. Drosten explicitly named that an increase in CT from 25-27 across the border of 28 means that individuals from whom these smears were obtained with the higher CT are no longer infectious. "*And again, you see a Ct shift from 25 to 27 approximately, 27, 28. And that's a range where, in our estimation, that's really where infectivity ends. If you see such a patient sample and you would ask, is the patient still infectious, I would say: No, this is now slowly no longer an infectious area. You can correlate that*." page 4 (top right column in: https://www.ndr.de/nachrichten/info/coronaskript270.pdf)

With these CT data, C. Drosten presumably refers mainly to a study on vaccine efficacy in Israel, which was verified by RT-qPCR. This study, which was available in a preprint publication ("Decreased SARS-CoV-2 viral load following vaccination" (https://www.medrxiv.org/content/10.1101/2021.02.06.21251283v1) and in the meantime is available regularly under the title " Initial report of decreased SARS-CoV-2 viral load after inoculation with the BNT162b2 vaccine (https://www.nature.com/articles/s41591-021-01316-7") is also referred to in a letter from the German Robert Koch Institute (RKI; AZ: ID3176 of 31.03.2021) to the German Federal Ministry of Health. In this study, PCR tests after vaccination (with BNT162b2) show that in vaccinated subjects who become positive for SARS-CoV-2 in the PCR from day 12 after the first vaccination, the CT for the three tested genes (E, N, RdRp using the Seegene Allplex test kit, which has a specificity of 96-98.4 according to the Instant EQA scheme 340) increases from a mean CT of 25 to a mean CT of 27.

Compared with a similarly SARS-CoV-2 PCR positive unvaccinated cohort, this study establishes vaccination success based on a CT decrease of 1.64-2.33. „*Finally, applied on all infections (post-vaccination and unvaccinated, n=5,794), a multivariate linear regression model accounting for age, sex and vaccination quantify* ***Ct regression coefficients ranging from 1.64 (N gene) to 2.33 (RdRp) for vaccination after 12 days*** *or longer prior to infection sampling*", which is arithmetically equivalent to a 4-fold reduction in viral load in vaccinated versus unvaccinated. In the original: : „*As a difference of 1 Ct unit is equivalent to a factor of about 1.94 in viral particles per sample, these Ct differences represent a viral load ratio ranging from 2.96 to 4.68.*"

It is also noteworthy in the PCR analyses description, that the CT values up to 40 were analyzed and evaluated in this work (Extended Data Figure 4).

Accordingly, in a new recommendation dated April 16, 2021, the CDC also addresses the CT in SARS-CoV-2 PCR to the effect that it should have a value of no more than 28 in order to send PCR products from "vaccine breakthroughs" (i.e., RT-qPCR positive individuals after complete vaccination) to the laboratory for sequencing (https://www.cdc.gov/vaccines/covid-19/downloads/Information-for-laboratories-COVID-vaccine-breakthrough-case-investigation.pdf).

Also, a study from South Korea mentions a **CT of ≤ 25** as the upper limit of clinically relevant "positives" and uses this value for comparison with the goodness of antigen tests.

Original quote: " [....] based on a clinically significant Ct value of ≤ 25 (....)" (p. 3 in https://jkms.org/DOIx.php?id=10.3346/jkms.2021.36.e101).

Unanimous scientific opinion (including Dr. Fauci of the US CDC, but also a number of scientists quoted in the New York Times in August 2020, https://www.nytimes.com/2020/08/29/health/coronavirus-testing.html) is **that all "positive" results detected only after a cycle of 35 have no scientific (i.e.: no evidence-based) basis**. In contrast, the RT-qPCR test for the detection of SARS-CoV-2, propagated worldwide with the help of the WHO, was (and following it all other tests based on it as a blueprint) set to 45 cycles without defining a CT value for "positive".

Also as early as May 2020, a position paper was issued by the National Centre for Infectious Disease in Singapore (https://www.ncid.sg/Documents/Period%20of%20Infectivity%20Position%20Statementv2.pdf), which points out that.

1. it is important to note that viral RNA detection by PCR does not equate to infectiousness or viable virus
2. the cycle threshold value (CT) of the PCR, as a surrogate marker for the viral RNA content, already detects viral RNA from a CT of 30, but no longer the presence of replicable viruses and the persons concerned are not infectious.

Original text extract "*6. A surrogate marker of 'viral load' with PCR is the cycle threshold value (Ct). A low Ct value indicates a high viral RNA amount, and vice versa. As noted above,* ***detection of viral RNA does not necessarily mean the presence of infectious or viable virus****. In a local study from a multicenter cohort of 73 COVID-19 patients***,** *when the Ct value was 30 or higher (i.e. when viral load is low), no viable virus (based on being able to culture the virus) has been found.*"

The RKI also states on its homepage as of 11.08.2020. (https://www.rki.de/DE/Content/InfAZ/N/Neuartiges_Coronavirus/Vorl_Testung_nCoV.html#doc13490982bodyText4) "*First results from diagnostics at RKI show that loss of cultivability in cell culture was associated with an RNA amount of <250 copies/5 µL RNA determined by real-time PCR (note: is RT-qPCR). This RNA level corresponded to a Ct value >30 in the test system used.*"

A recent study from South Korea (https://www.nejm.org/doi/full/10.1056/NEJMc2027040) **sets the limit for virus cultivability at a CT value of 28.4.**

In another recent study from Frankfurt (https://www.mdpi.com/2077-0383/10/2/328), it was shown that of 64 RT-qPCR positive patient samples (one gene tested), virus cultivation in cell culture was only possible from 33 (=52%). These infectious samples were already positive up to a mean CT value of 26 (Supplementary Figure 1), whereas virus cultivation was no longer possible from the samples with a higher CT.

In the round robin Instant e.V. (http://www.finddx.org/covid-19/pipeline/?section=molecular-assays#diag_tab.) see also next point, the enormous range of CT values even for highly standardized samples between the different laboratories and also with regard to the different target genes becomes apparent. For example, here the CT for the same defined diluted sample of SARS-CoV-2 (sample number 340061) for the WHO-recommended genes varies between 15-40 (E gene), 20-40.7 (N gene) and 19.5-42.8 (RdRp gene). **This impressively demonstrates an extreme lack of test standardization within the participating (and certified) laboratories.**

Against this background, it is disconcerting when RT-qPCR is still considered the "gold standard" by the RKI (and WHO) without defining the exact validations and external certification conditions (and without these apparently being fully monitored by the authorities).

Assessment:

In general, RT-qPCR cannot detect intact, propagable (infectious) viruses, not even the complete intact viral genome, but only nucleic acid of the sought section. In principle, it is possible to define a threshold (CT), above which a positive PCR signal no longer correlates with replication viruses, by validation with a parallel virus cultivation in cell culture for well adjusted and correctly performed PCR tests. This has been a well-practiced routine in blood product monitoring (for HIV and Hepatitis viruses) for years.

This stringent validation then allows - as long as the test system is NOT changed - as a surrogate marker an estimation of the viral load and thus the possible infectivity of the tested sample, but never definitive detection. As soon as a component of the PCR test system (be it chemicals, plastic goods, enzymes, protocol procedures or machines) is changed in one of the applied steps, it is mandatory to recalibrate the system.

From all the information published so far (see above), it can be assumed that any CT value above 35 is no longer associated with the cultivability of infectious viruses and is therefore the absolute threshold for the decision "positive", also irrespective of the test system used. The CT range 25-35 may still be validly assessed as "positive in the sense of infectivity" in a test-dependent manner if, as described, it has been compared with a virus cultivation by adequate validation in the performing laboratory.

CT≤ 25 : positive

CT 26-35 : positive only if matched with viral culture

CT > 35 : negative

The strict evaluation of the CT value mainly matters when the target number is one, but generally applies to each individual target.

The threshold CT 25 was already introduced in December 2020 by the English "Office of national statistics (ONS), here with CT above 25 as negative. Table sheet 2 (Data) in the linked Excel data sheet (link below). Results: " *„The analysis shows: - People with a higher concentration of viral genetic material (positive cases with low Ct values; below 25) are more likely to be infectious in a household than those with lower concentrations (positive cases with high Ct values; above 25).”*
(https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/

conditionsanddiseases/adhocs/12683coronaviruscovid19infectionsurveycyclethresholdandhouseholdtransmissionanalysis).

With reference to this ONS threshold, the authors of a large cohort study from Münster, Germany (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8166461/), using RT-qPCR of the ORF-1ab and E genes in smear samples from 162457 individuals, concluded that : „*RT-PCR test positivity should not be taken as an accurate measure of infectious SARS-CoV-2 incidence*“.

This study showed that a total of 2.6% of the samples had a positive RT-qPCR result. The CT threshold above which the samples were considered definitely negative was set at 40. The samples were also analyzed according to the number of samples that became positive up to a cutoff value of 25 (always both genes, personal information on request to A. Spelsberg).

The results showed that in asymptomatic individuals only 0.4% (68 of 16,874 individuals) had a positive RT-qPCR test with a mean CT of almost 29. Of these, only 27% (= 18 individuals) had a CT of up to 25, which was considered by the authors “*indicating a likelihood of the person being infectious*“. Converted, this means that in only 18 of 16874 (=0.1%) asymptomatic (healthy) individuals did PCR indicate possible infectivity with respect to SARS-CoV-2.

Also, of 6212 symptomatic individuals from the peak periods of the first two "Corona waves," only 403 individuals (=6.5%) had a positive RT-qPCR for SARS-CoV-2 with a mean CT of 27.8 (1st wave) and 26.6 (2nd wave). Of these positives, a maximum (in the 2nd wave) of 40% (= 145/367) and in the first wave even only 26.5% (=10/36) individuals had a CT of up to 25 and could thus be classified as probably infectious. Consequently, only 155 of 6212 symptomatic (ill) persons (=2.5%) could be assumed to be possibly infectious with SARS-CoV-2.

Values from Table 1 of: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8166461/

In this respect, it can be assumed that all CT values higher than 30 may no longer be used to assess whether the person from whom the sample was obtained "is infectious", but rather the UK ONS CT of 25 must be used.

However, taken on its own, without information about the matching with the specific number of viral genomes (viral load) and the correlation with a cultivability of corresponding virus quantities, the CT value even at low numbers is worthless as an evaluation criterion of a positive PCR detection.

1.3.3 Adequate controls

In order to **correctly assess sensitivity and specificity of an RT-qPCR**, adequate samples must be included in each reaction run. This begins at the test site with "**blank swabs**" to reliably exclude contamination at the sample collection site, continues with **extraction controls** to ensure the correct isolation of reproducible RNA with all subsequent processing steps, i.e. an artificially produced defined RNA which is carried and processed in all steps of sample preparation up to PCR and for which PCR is then also carried out with the aid of suitable primers. This excludes the possibility of inhibitory substances or errors preventing the amplification of RNA during sample processing.

Such defined controls have been available through Instant e.V. since November 2020. From the booklet accompanying the shipment of the defined (https://www.instand-ev.de/fileadmin/uploads/user_upload/Dokumente/Virologie/20210118g_Begleitheft_-_quantitative_Bezugsproben_1_und_2_-_SARS-CoV-2.pdf), the following aspects can generally be identified:

- The strain BetaCoV/Munich/ChVir984/2020 was used as a control as a heat-inactivated sample with controlled viral counts corresponding to 10^6 and 10^7 RNA copies/ml, since this was the threshold for assessing patients as "probably contagious" (in: 2.2. Intended use). According to the database (https://www.european-virus-archive.com/virus/human-2019-ncov-isolate), this strain was obtained in Munich on 28.01.2020 and is sold via Charité.

- Depending on the tested genes and the performing laboratory, a wide range of CT values was shown despite defined sample in reference laboratories. For example, this CT value varied for the E gene for the sample with 106 copies/ml between 21.9 (lab 4) and 28.7 (lab 1) and the RdRp gene between 24.8 (lab 4) and 33.0 (lab 1) with the N gene already at a CT of 22.1. Across all tests from reporting laboratories, there was a spread of CT values of 12-38 for this sample (Figure 2) and a spread of CT values of 10-36 for the higher concentration sample. This example alone shows that each laboratory must always carry the defined samples in each test series in order to be able to convert the laboratory's own CT value to the viral load, which should be the actual reference sample for assessing the tested patient sample.

The extreme variation of CT values in the different test systems is addressed by C. Drosten in his NDR Podcast 94 of June 22, 2021 (https://www.ndr.de/nachrichten/info/coronaskript306.pdf) as follows: "*And namely the Ct values that we have here, they are not easily comparable between the individual test manufacturers."...." But we can only compare them numerically as long as we are in the same test system. The differences there are sometimes considerable. There are test manufacturers for whom a value of, say, 25 is nothing at all worrying, while the same value of 25 in another manufacturer's test shows that this is already a seriously infectious concentration. That's simply because these test manufacturers don't standardize on the Ct value*." (pg. 17) He further complains that this standardization does not take place with calibrations (produced by him) (pg.18): "*What does not happen at the moment, however, is that nationwide recommendations are also made and applied by the state health offices or also by the Robert Koch Institute for certain areas of application on this technical laboratory basis that has now been created*." Means: since autumn 2020, suitable controls for virus load determination would be available and would have to be requested by the authorities for the laboratories to validate the test results, but this is obviously not happening (according to C. Drosten). "***We can even do it in such a way that this inherent problem of non-comparability of Ct values is already solved. Mind you, in the fall. The technique and the lab testing is not the catch here, but it's again the implementation and the regulation***."

Furthermore, each correct test series must include **a series of external negative controls** (i.e., carried in parallel as patient samples) and a positive control, ideally consisting of an inactivated defined SARS-CoV-2 virus strain. This would be an original task of the RKI an other official government organizations like the CDC or WHO (with the assistance of other suitable public institutions such as the Bernhard Nocht Institute or the Friedrich-Löffler Institute in Germany) to isolate a sufficient number of SARS-CoV-2 viruses from patient samples in the laboratory facilities available there (safety level 4). Those institutions then could cultivate defined strains from these as controls, to inactivate these and to deliver them in defined virus numbers as controls to the testing laboratories via the local supervisory authorities. However, since this important service is still not offered even after more than a year of the "pandemic", the positive control usually consists of a synthetic RNA that only encodes the target genes of the test system. This positive control can also be used to determine the lower detection limit of the PCR. This is specified by some commercial kits as 20 or fewer viral genomes per sample and thus (see point 1.3.2.) already detects a virus quantity in the smear that is below the infectious dose by a factor of 10^5, i.e. has no diagnostic/prognostic value whatsoever. An overview of the currently used commercial kits with their line data can be found at http://www.finddx.org/covid-19/pipeline/?section=molecular-assays#diag_tab.

Interlaboratory tests:

Correctly performed controls also include the participation of the laboratories performing the tests in so-called "interlaboratory comparisons" (see also 1.3.1.). In these, an anonymized panel of test samples is made available by an external provider. In the case of virus detection, these contain negative samples and samples with closely related viruses (inactivated) to check the specificity (these samples must not give a positive signal) and positive samples with different dilutions of the virus sought (inactivated) to determine the sensitivity (from which number of viruses does the PCR become positive, with which CT value).

In the case of SARS-CoV-2, the first EQA scheme "Virus Genome Detection - SARS-CoV-2 (340)" by the association "INSTANT e.V." was ready in April 2020. According to the report, 488 laboratories participated in this EQA scheme, of which 463 reported results. The results can be read in the published commentary (Zeichhardt M: “Kommentar zum Extra Ringversuch Gruppe 340 Virusgenom-Nachweis SARS-CoV-2", available at: https://corona-ausschuss.de/wp-content/uploads/2020/07/Instand-Ringversuch-Virusgenom-Nachweis-SARS-CoV-2.pdf) and show two deviations from the usual EQA procedure, which already here pointed to laboratory problems with RT-qPCR for the detection of SARS-CoV-2:

For example, page four of the publication states, "*Important evaluation notice: only 4 of the 7 samples tested in this Extra EQAS will be considered for obtaining a certificate of successful participation*." The footnote on page 10 of the commentary states, "*In the April 17, 2020 interim evaluation, all participants in the Extra INSTAND EQA trial (340) Virus Genome Detection of SARS-CoV-2 April 2020 were notified ahead of time of the sample characteristics of samples 340059, 340060, and 340064. The results of these 3 samples will not be considered for the granting of a certificate* [....]" The reason for this exclusion of certain samples is explained on page 4 of the commentary: "*While the extra ring trial was still running, INSTAND e.V. received urgent requests from Germany and abroad to reveal the properties of the samples to be tested before the end of the extended submission period, i.e., before April 28, 2020, so that laboratories can improve their test method in the short term in case of possible incorrect measurements*." (page 4 above in INSTANT e.V. report))

This procedure is very unusual for a real round robin test and thus no longer represents an independent external verification procedure of the participating laboratories.

Despite the samples already detected and the reduced test scope, sample mix-ups occurred in a large number of laboratories - as stated on page 18 of the commentary: "*For sample 340064 (SARS-CoV-2 positive diluted 1 : 100 000), the reduced success rate of only 93.2 % is essentially based on incorrect result assignments (mix-ups) for sample 340064 and sample 340065 (negative for SARS-CoV-2 and positive for HCoV 229E). The mix-ups for samples 340064 and 340065 involved 24 laboratories with a total of 59 results per sample. See also section 2.4.2.1* [...]." Thus, a large number of laboratories mistakenly confused sample 340064 (slightly diluted SARS-CoV-2) with sample 340065 (negative for SARS-CoV-2 and positive for the closely related virus HCoV 229E).

Apart from the startling fact that a considerable number of samples were obviously mixed up even under highly standardized procedures in an interlaboratory comparison (which raises the question of the corresponding rate of sample mix-ups and thus wrongly assigned swab samples under mass testing conditions), it is striking that all reported mix-ups concerned only these two samples, but not the samples with the final numbers 61 (very highly diluted SARS-CoV-2) and 62 (negative), which were also evaluated. The detailed results of a second round robin test from June/July 2020 (https://www.instand-ev.de/System/rv-files/Zusammenfassung%20der%20Probeneigenschaften%20und%20Sollwerte%20Virologie%20340%20Juni%20Juli%202020%2020200911a.pdf) are still not publicly available.

1.3.4 Exclusion of contaminations of reagents and "problems in the course of action".

The best PCR design can still lead to false positive results if either the underlying reagents / kits are contaminated with positive samples or, much more likely, contamination occurs in the laboratory workflow. Since PCR is an extremely sensitive method (exponential reaction course) that can detect few molecules of a DNA, laboratory contamination by PCR end products is a major problem in clinical diagnostics (described e.g. already in 2004 in Aslanuadeh J et al., http://www.annclinlabsci.org/content/34/4/389.full.pdf+html: „*A typical PCR generates as many as 10^9 copies of target sequence and if aerosolized, even the smallest aerosol will contain as many as 10^6 amplification products [6]. If uncontrolled, within a relatively short time the buildup of aerosolized amplification products will contaminate laboratory reagents, equipment, and ventilation systems [6]*.)

This extreme risk of contamination requires that the diagnostic laboratories working with PCR take the utmost care in testing - very competent staff, contamination-proof environment, permanent independent control.

Already in the above mentioned round robin 340 in April a problem with false positive results appeared, which was commented as follows (page 20 below): "*In addition, in some cases the tests with the SARS-CoV-2 negative control samples 340060, 340062 and 340065 indicate specificity problems, which are independent of mix-ups of the samples 340064 and 340065. Clarification is needed as to whether these false positives are due to a specificity problem with the tests used or to carryover of SARS-CoV-2 during test performance or to mix-ups with other samples in this EQA at the laboratories in question*." (page 21 bottom in https://www.instand-ev.de/System/rv-files/340%20DE%20SARS-CoV-2%20Genom%20April%202020%2020200502j.pdf). For mix-up in this EQA scheme, see details point 3.3. end of paragraph.

If, against this background, one further sees how, for example, according to a BBC report, work is carried out openly and extremely contamination-prone with untrained personnel in large test laboratories in England (https://www.youtube.com/watch?v=Uk1VK1reNtE), it is not surprising if even in Germany and other countries (where such reports have not yet been filmed) occasional reports of "false positive cases" due to laboratory contamination are found in the media (e.g. MVZ Augsburg - link at the end of the section). Even under controlled laboratory conditions, contamination due to PCR steps cannot be safely excluded in such a highly sensitive method. Thus, the problem of false positive PCR results in SARS-CoV-2 diagnostics due to laboratory procedures and already pointed out in the first publication of RT-qPCR (Corman et al., 10.2807/1560-7917.ES.2020.25.3.2000045): „*In four individual test reactions, weak initial reactivity was seen but they were negative upon retesting with the same assay*“ [.....] „.... *most probably to handling issues*....“

Even if the course of action in the laboratory functions optimally and is extremely monitored in order to greatly minimize laboratory-related contamination, an unexpected source of false positive results can arise here in the **contamination of the materials/chemicals used ex-manufacturer**. For example, the swab materials used to take samples may already be contaminated ex works - as in the case of the "Phantom of Heilbronn", in which the cotton swabs used to take DNA traces at the crime scenes were contaminated with the DNA of a packaging worker from the manufacturer's plant, thus hampering forensics with false traces for years (https://www.faz.net/aktuell/gesellschaft/kriminalitaet/dna-ermittlungspanne-das-phantom-von-heilbronn-ist-widerlegt-1925411.html).

In the case of SARS-CoV-2 diagnostics, a contamination problem due to PCR primers containing positive controls ex works was also published in June 2020 (Wernike et al., DOI: 10.1111/tbed.13684). Here, it had been noticed that even pure water samples with several independent primer batches gave unambiguous positive SARS-CoV-2 detection in RT-qPCR: "*However, there were also primers/sample sets that displayed very low-level contaminations, which were detected only during thorough internal validation*."

Also, some false-positive results of SARS-CoV-2 RT-qPCR testing reported in the daily press in summer 2020 were attributed to material issues (e.g., https://www.br.de/nachrichten/bayern/probleme-in-augsburger-labor-bringen-falsche-testergebnisse,SEh5Qq4) and e.g. in the United States where 77 football players were tested positive for SARS-CoV-2 and then a second confirming test revealed that the results of the first tests were all false positive (https://www.nfl.com/news/all-77-false-positive-covid-19-tests-come-back-negative-upon-reruns)

Evaluation:

Even with ideal RT-qPCR design and good laboratory practice with adequate validation, problems in daily handling procedures as well as externally via samples already contaminated ex-factory can significantly influence the quality of results of RT-qPCR and lead to false positive results.

1.3.5 Commercial PCR test kits: Approval for diagnostics?

Very early on, commercial PCR test systems, the "PCR kits", were used in routine laboratories for diagnostics, although the majority of them were declared for "RUO" ("research use only").

The first, and therefore most concise test manufacturer, the Berlin (Germany) company TIB Molbiol, whose company owner (Olfert Landt) was already listed as author on the WHO protocol recommendations alongside Christian Drosten, deserves special mention. The kits, which are accordingly based on the WHO recommendations, are used via the company Roche on their large-scale automatic machines "Cobas" and should therefore make up a large percentage of the kits used for routine diagnostics of SARS-CoV-2 in Germany and around the world.

Exact figures cannot be determined, however, TIB Molbiol has already delivered more than 60 million of these tests worldwide in 2020 according to its own information (https://www.tib-molbiol.de/de/covid-19), although these are still declared as "***Not tested for use in diagnostic procedures***" (e.g. header in https://www.roche-as.es/lm_pdf/MDx_53-0777_96_Wuhan-R-gene_V200204_09155376001%20%282%29.pdf). The corresponding package inserts with the protocol information and kit descriptions of the company TIB Molbiol were astonishingly according to metadata of the originally available PDFs (can be provided electronically) already on 15.01.2020 (!!!) completely with ROCHE SAP number are still available unchanged (albeit with metadata analysis 06.02.2020) parallel to other test kits, which now have an approval for in vitro diagnostics.

In the meantime (as of July 2021), there is a wide range of PCR detection systems (https://www.theglobalfund.org/media/9629/covid19_diagnosticproducts_list_en.pdf), many of which are also approved for in vitro diagnostics (IVD) of SARS-CoV-2. (e.g. here: https://www.genesig.com/assets/files/Path_COVID_19_CE_STED_IFU_Issue_500.pdf). In the description of these kits it reads under 1. „Intended use: "***Positive results are indicative of the presence of SARS-CoV-2 RNA***. *Positive results do not rule out co-infection with other bacteriaor other viruses. Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions.* ***Positive and Negative results must be combined with clinical observations, patient history, and epidemiological information***“

1.4 Relationship between positive nucleic acid detection in RT-qPCR, disease and infectivity

Only those actually infected can pass on the virus and carry the risk of disease, and thus should be used to determine the progression of an infection rate and wave of disease

"*PCR detection is the standard test for diagnosing viral infections such as SARS-CoV-2. The test detects individual pathogen genes but not intact pathogens*." And, "*There is a possibility that the test will be positive beyond the duration of infection because "viral debris" is still present in the nose or throat. Reliable proof of infectivity is only possible with elaborate tests that involve laboratory testing to determine whether the material from the swabs can kill living cells*." This was written by the German medical journal "Dt. Ärzteblatt" on 02/01/2021 (https://www.aerzteblatt.de/nachrichten/120745). Also, the CDC points out under "Disadvantages" of NAATs (nucleic acid amplification tests = PCR) "**A positive NAAT diagnostic test should not be repeated within 90 days, because people may continue to have detectable RNA after risk of transmission has passed**" (below in summary table at: https://www.cdc.gov/coronavirus/2019-ncov/lab/resources/antigen-tests-guidelines.html#previous)

"*The PCR assay detects gene segments of SARS-CoV-2; it does not tell us whether they are infectious viruses or viral remnants after passed through infection. This would require pathogen culturing*." Was stated in an August 2020 publication by the head of Frankfurt's public health department (https://www.laekh.de/fileadmin/user_upload/Heftarchiv/Einzelartikel/2020/10_2020/Die_Covid-19-Pandemie_in_Frankfurt_am_Main.pdf). And in his expert opinion of April 21 for a court in Heidelberg (to be viewed anonymously here: https://www.corodok.de/wp-content/uploads/2021/05/Gutachten-Prof.-Drosten-v.-31.3.2021-anonymisiert.pdf), the expert C. Drosten confirms that an RT-PCR test can also be positive if "*at least the section to be detected from the genome of the virus is present in the tested sample*". This means that genetic material fragments can also yield positive results in the PCR without originating from an intact, replication competent virus, thus also providing an alleged virus detection in non-infectious samples.

In a CDC publication dated 7/13/20 titled "*CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel For Emergency Use Only Instructions for Use*", (https://www.fda.gov/media/134922/download), on p. 38 under the heading "*Limitations*" (still found on p. 37) : "***• Detection of viral RNA may not indicate the presence of infectious virus or that 2019-nCoV is the causative agent for clinical symptoms.***"

That a mRNA detection of SARS-CoV-2 does not necessarily correlate with disease and should not be used as the sole criterion for disease assessment, but is only an aid to confirm a clinical diagnosis, is also clearly described in WHO Information "*Notice for IVD Users 2020/05, Nucleic acid testing (NAT) technologies that use polymerase chain reaction (PCR) for detection of SARS-CoV-2*" dated Jan. 13, 2021 (published Jan. 20, 2021 at https://www.who.int/news/item/20-01-2021-who-information-notice-for-ivd-users-2020-05): **: „***Where test results do not correspond with the clinical presentation, a new specimen should be taken and retested using the same or different NAT technology.”*

Further: : “*Most PCR assays are indicated as an aid for diagnosis, therefore, health care providers must consider any result in combination with timing of sampling, specimen type, assay specifics, clinical observations, patient history, confirmed status of any contacts, and epidemiological information*”

Also in a recent publication in Lancet (https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(21)00425-6/fulltext#%20), the authors refer to the RT-qPCR assay as follows: : „***In our view, current PCR testing is therefore not the appropriate gold standard for evaluating a SARS-CoV-2 public health test***”, because, in their opinion, the PCR still comes up positive even when those tested are no longer positive, since the RNA can persist in the body for weeks and months even after the immune system has successfully combated it, without the person still being infectious. „*Once SARS-CoV-2 replication has been controlled by the immune system, RNA levels detectable by PCR on respiratory secretions fall to very low levels when individuals are much less likely to infect others.* ***The remaining RNA copies can take weeks, or occasionally months, to clear, during which time PCR remains positive”***

In a May 2021 publication in Science led by C. Drosten (DOI: 10.1126/science.abi5273) examining the infectivity of SARS-CoV-2, the authors define in the very first sentence of the abstract the **parameters for quantifying and potentially passing on the virus as „...** ***viral load and whether samples yield a replicating virus isolate in cell culture*.**“ They further state in the introduction that viral load is determined by viral RNA concentration **and successful virus isolation in cell culture assays**. Furthermore, they point out that even “.... *viral load and cell culture infectivity cannot be translated directly to in vivo infectiousness, and the impact of social context and behavior on transmission is very high, these quantifiable parameters can generally be expected to be those most closely associated with transmission likelihood*”.

In his NDR Podcast 94 of June 22, 2021 (https://www.ndr.de/nachrichten/info/coronaskript306.pdf) page 16, C. Drosten addresses the relationship between CT value and infectivity as follows: *"....that a case just because the* ***patient has a high CT value at this moment, that is, because he may not be infectious right now****, so he has little virus, he has virus, but he has little virus,....*"

1.5 Conclusion: Significance of RT-qPCR Tests for the Detection of SARS-CoV-2 Coronavirus Infection

In view of the problems outlined in section 1.3, RT-qPCR is not a suitable and reliable (and approved) diagnostic tool for the detection of infectious (replication capable) SARS-CoV-2 viruses.

Furthermore, the sole RT-qPCR test result is only a laboratory value, which, in view of the aspect outlined in point 1.4, does not permit any statement about the presence of infectious viruses and may only be used at all in conjunction with a clinical symptom diagnosis (ascertained by healthcare providers, in Germany medical doctors).

Summary: RT-qPCR is not suitable for the detection of SARS-CoV-2 infection in asymptomatic individuals by means of a nasopharyngeal swab, as is done uncritically in large numbers and predominantly by non-medical personnel WITHOUT (crucially here: contrary to the WHO requirement!) taking a medical history and ascertaining the symptoms of those tested.

2. Antigen detection by means of a rapid test

2.1 Explanation of terms/basics of the rapid test

The "rapid tests" currently used for the diagnosis of SARS-CoV-2 are based on the principle of an antigen test according to the "lateral flow" test procedure. This detects a protein component (protein) of the virus.

An **antigen** is a three-dimensional structure of proteins and other organic materials that can be recognized and bound by antibodies (immunoglobulins).

In the case of **viral antigens**, these are usually individual protein components (proteins) from the viral structure. These can be either complete structural proteins such as the "spike" protein located on the surface (S protein, these are the "stalked buttons" in the virus drawings) or the envelope protein ("envelope" - E protein) or that protein from which the nuclear envelope is built (nucleocapsid = N protein). Fragments of these complete structural proteins are also often sufficient to be bound by antibodies. These are the so-called **epitopes**, which also represent the actual antibody binding site on the intact structural protein. Each structural protein usually has a large number of epitopes, so that different antibodies can bind simultaneously to different epitopes of the same protein.

In case of SARS-CoV-2, the major antigens (the above-mentioned, S, E, and N proteins) are those that trigger an immune response in the body when infected with the virus. As a result, the body forms antibodies that specifically recognize these antigens, then bind to them (**antigen-antibody reaction**) to neutralize the viruses and render them destructible to immune cells.

This antigen-antibody reaction can be used in the laboratory to search for the antigens in any sample with synthetically produced antibodies.

The basic principle of the so-called **antigen tests** in the laboratory (these aim at the detection of antigens by antibodies, unlike RT-PCR, which detects nucleic acids) is that two matching antibodies are produced in vitro, which recognize two different epitopes of the antigen being searched for, a so-called "**antibody pair**". Both antibodies must be selected in such a way that they can only recognize and bind the desired epitope on the antigen sought, but not other structures on similar antigens. They must therefore be highly specific in order to be used in diagnostics. This **high specificity** of diagnostic antibodies is ensured in test development by matching them with many very similar epitopes. All antibodies that bind undesired epitopes are discarded until only one ideal antibody pair remains that meets the requirements of very high specificity, high binding property (sensitivity) and no mutual interference.

The antigen test is then built on this antibody pair, in which the antigen sought is bound by both antibodies simultaneously and is sandwiched between them like the fry inside the sandwich bun (hence "**sandwich test**").

For the lateral flow rapid antigen tests, which are currently used in broad-spectrum population testing for the detection of SARS-CoV-2 antigens, this sandwich test system is now used.

Here, the first of the two specific antibodies is bound to a carrier material in such a way that its antigen binding site points freely upwards. This is the later region in the rapid test where a color change gives the signal "positive". The second antibody is coupled with a detection system that is later responsible for the color reaction and is located as a depot directly next to the site in the rapid test at which the sample is dripped on.

Test procedure: If the antigen, in this case the protein of SARS-CoV-2, is present in the swab sample, it binds with the first specific antibody from the depot after dropping into the test field of the detection cassette. Capillary forces cause the mixture of antigen with bound first antibody and excess unbound antibody to migrate from the depot towards the test field. Here, the second specific antibody fixed there then binds the antigen with the first antibody already bound to it. The solution migrates beyond the test field over another field where the excess antibodies are captured (control field). The detection system of the test begins to show a **chemical color reaction** wherever the first antibodies are bound. In the control field, this is caused by the surplus first antibodies that are now bound here and have "brought along" the detection system, thus indicating that the test has in principle functioned without interference.

In the test field, there is only a color change if an antigen was actually in the sample and was bound via the second antibody fixed there. Since the antigen has already arrived at the test field with the first antibody and the detection system, the chemical color reaction also begins here, which leads to the color change (usually a violet stripe) at the test region.

Whenever the antigen sought is present in the swab sample, it can bind the first antibody and transport it together with the detection system to the fixed second antibody, which then intercepts this antigen-antibody-detection system complex and thus causes the positive signal at this point.

The color change at the test site (**signal "positive"**), which causes the visible stripes in the rapid test, is a chemical reaction and therefore can be influenced by reaction conditions such as pH or chemicals that come with the sample and is a clear weakness in the reliability of the test.

This explains the many videos circulating on the internet that detect SAR-CoV-2 using the rapid antigen tests in apple juice, red wine, beer, etc.

2.2 Basic information on the diagnostic significance of the rapid antigen test

Like RT-PCR, rapid antigen tests cannot in principle determine whether the viral antigen found belongs to an intact, infectious virus or is a remnant (fragment) of viruses that have been killed by the immune system.

Irrespective of this general limitation of the significance with regard to infectivity, rapid tests only have an indicative character, not a reliable diagnostic significance.

The most well-known rapid test before Corona times was the rapid pregnancy test, which works according to the same principle of the antibody-antigen test. However, here the pregnancy hormone (HCG) acts as an antigen. If this is present in sufficient quantity in the tested urine, the test indicates "positive" - in this case, presumably pregnant. However, the rapid test alone will never be sufficient as a well-founded proof of pregnancy; in this case, the doctor will use HCG detection in the blood as well as an ultrasound to make the diagnosis.

The rapid antigen tests for the detection of SARS-CoV-2 components can also only give an indication of possible colonization or infectivity and are subject to similar limitations as RT-qPCR.

2.3 Factors influencing the reliability of rapid antigen tests

2.3.1 Pre-test probability

In an infographic entitled "Understanding Corona rapid test results" (https://www.rki.de/DE/Content/InfAZ/N/Neuartiges_Coronavirus/Infografik_Antigentest_PDF.pdf?__blob=publicationFile), the RKI clearly explains how the probability that a test result is correct depends on the so-called **pre-test probability**, i.e., on the actual number of genuinely infected persons in the tested population. This aspect of pre-test probability applies to both the rapid antigen tests and equally to the RT-qPCR tests.

The calculation example presented by the RKI for the interpretation of the rapid antigen tests assumes a realistic scenario starting from a sensitivity (susceptibility) of the antigen tests of 80% and a specificity (reliability) of 98%, whereby it is also explicitly mentioned here (https://www.rki.de/DE/Content/InfAZ/N/Neuartiges_Coronavirus/Vorl_Testung_nCoV.html): "*The considerable differences in performance of the various commercially available tests must be taken into account here* (reference to: https://www.medrxiv.org/content/10.1101/2020.10.01.20203836v1)."

Assuming fife persons out of 10,000 tested are truly infected with SARS-CoV-2, 200 false positive tests and four true positive tests will still show up. This means that one truly infected person per 10,000 would be missed, but 200 would get a false positive result and therefore have to be quarantined/isolated until RT-qPCR testing then gives the "all clear". This would mean in the case of a school test with e.g. 1000 students, that 20 would get a false "You are Corona positive" and the school would first be closed as an "outbreak site" until the retesting by RT-qPCR gives the all-clear. Such cases have already been reported in the press.

-For example, in Altdorf near Nuremberg (Germany), 29 of 180 high school students tested positive in a rapid antigen test; upon examination, 28 of them turned out to be negative (https://www.merkur.de/bayern/nuernberg/nuernberg-corona-bayern-test-fiasko-schnelltests-fehlerhaft-positiv-schule-altdorf-gymnasium-zr-90253265.html).

- In Potsdam (Germany), 12 of 36 teachers tested positive with a rapid antigen test and were sent into quarantine. After review, all test results turned out to be false positives (https://www.news4teachers.de/2021/03/sorgen-schnelltests-fuer-chaos-an-schulen-falscher-alarm-legt-grundschule-lahm/).

- Medscape even headlined, "*200 false positives, 8 detected, 2 missed - why pediatric and adolescent physicians are skeptical of mass rapid testing* (https://deutsch.medscape.com/artikelansicht/4909842)

And even if the rate of genuinely infected persons in the tested group were very high, as in the second calculation example from the RKI (with 1000 out of 10,000 tested persons), the hit rate of the rapid tests would be poor and 180 persons would receive a false positive result and 200 a false negative test. This is where the poor sensitivity of the test comes into play.

In the "*Hinweisen zur Bewertung der Ergebnisse aus AG-Testen*" (Note: Antigen rapid tests, *Notes on the evaluation of results from AG tests*) on the webpage of the RKI, the problem of false positive antigen tests is addressed: "*A positive test result by means of AG test triggers the suspicion of a transmission-relevant infection with the SARS-CoV-2 and requires a follow-up test by means of PCR to avoid false positive results. In view of the potentially significant consequences of incorrect results, there are high requirements not only for the sensitivity of antigen tests, but also for their specificity. Thus, with low prevalence/pretest probability and low test specificity, a high number of false-positive results and a corresponding additional burden on the ÖGD due to imposition and, if necessary, withdrawal of measures would have to be expected.*" https://www.rki.de/DE/Content/InfAZ/N/Neuartiges_Coronavirus/Vorl_Testung_nCoV.html

2.3.2 Sensitivity

Due to the fact that in the antigen test there is no such strong (exponential) amplification of the output signal as in RT-qPCR, but only a limited signal amplification due to the chemical color reaction, **this type of test is significantly less sensitive than the RNA detection by RT-qPCR used for comparison**.

This "underperformance" of rapid antigen tests is the subject of a Lancet article (https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(21)00425-6/fulltext#%20), but here the negative test result in the rapid antigen test (here called LTF, lateral flow test) is relativized to: *„[....] in all six observed cases, viral loads were very low (Ct ≥29 reflecting around <1000 RNA copies per mL in the laboratory used)—when LFT should be negative*."

A brand new study from Norway (https://pubmed.ncbi.nlm.nih.gov/33736946/) confirms this finding that in asymptomatic individuals, rapid tests have an unsatisfactorily high inaccuracy, and that only in symptomatic individuals are reasonably accurate detections made of those who are actually infected. The authors conclude: „*Our results indicate that the test correctly* *identified most infectious individuals. Nevertheless, the sensitivity is considerably lower than for PCR“*

In a comparison of rapid antigen tests (3 different manufacturers) with unspecified RT-qPCR results ("different RT-qPCR methods" in the original: "*using different RT-qPCR methods*") in 5066 cases, of which 101 (=2%) had a positive RT-qPCR result, the parallel antigen test had a sensitivity of only 42.6%, with 16 false positive results (0.32%) and 58 false negative (1.15%) results, compared to RT-qPCR, in which CT values up to 35 were considered positive (corresponding to a converted viral load of 31 RNA copies/ml).

Here, positive antigen tests correlated very well with a high viral load (mean 2.7×10^6 copies/ml) and low CT (up to 22, Figure 4) and typical disease symptoms, with low viral load with less than 10^6 RNA copies/ml (note: considered to be the limit of infectivity, a.o. by the RKI) and especially asymptomatic individuals, the rapid tests were often negative, which is considered a deficiency in this publication (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8234263/pdf/main.pdf), but against the background of the identification of infectious individuals should be more reliable than RT-qPCR when evaluated with too high CT values.

Also C. Drosten explains in his podcast (No. 94 page 15) of22.06.2021 to the self-test (antigen test): "*There must be already proper virus to get the test positive. But there also has to be a good amount of virus to infect someone. This is a good match. Even in the thresholds, it's a good match. So we actually estimate that the infectivity threshold is about where the detection threshold of these antigen tests is.*" (https://www.ndr.de/nachrichten/info/coronaskript306.pdf)

This **alleged lack of sensitivity** is the most common criticism when the unreliability of rapid antigen tests is reported. For example, the Pharmaceutical Newspaper (https://www.pharmazeutische-zeitung.de/in-der-praxis-deutlich-unzuverlaessiger-als-auf-dem-papier-123017/) writes: "*Rapid antigen tests could detect mostly "highly infectious people with high viral loads," Keppler explains. "However, it is not the case that an infection could be reliably excluded by the negative result of a rapid test*." Here, however, the basis is comparing the rapid antigen test with RT-qPCR and criticizing the fact that only some of the RT-qPCR positive swab samples also become positive in the rapid antigen test.

For example, in Epidemiological Bulletin 3/2021, the RKI reports on a study using rapid tests in a Stuttgart clinic (from page 11 in: https://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2021/Ausgaben/03_21.pdf;jsessionid=15E8B09E615AECED77C34439BB8052AF.internet051?__blob=publicationFile). Here, Table 1 shows that of 18 RT-qPCR positive for SARS-CoV-2 RNA asymptomatic individuals, only 7 also had a positive signal in the rapid antigen test, and of symptomatic individuals, 36 of 42. Accordingly, the discussion states, "*Because of the very limited sensitivity of the antigen test in asymptomatic individuals, single testing in this population cannot adequately exclude SARS-CoV-2 infection.* ***Highly contagious individuals with low Ct values*** **(i.e., high viral load)** ***are detected with adequate confidence*.**" Here, the data show, "*At a Ct value of 22 or less, the detection rate of the antigen test was 100%*."

This example shows very clearly that a reliable antigen test, when performed correctly, correlates very well for symptomatic individuals with rapid response in RT-qPCR (low CT value), but not for asymptomatic, and only high CT value RT-qPCR positive, individuals. This speaks to the real-world significance of rapid antigen testing in terms of detecting a high viral load in symptomatic individuals. However, according to these data, the test is unsuitable for testing asymptomatic persons, both to reliably identify possibly infected persons and to reliably identify healthy persons as negative.

Such a finding was also obtained in the current Frankfurt study (https://www.mdpi.com/2077-0383/10/2/328), where three rapid antigen tests (there AG-RDT, antigen rapid diagnostic test) were matched with a viral culture from the same samples in cell culture and correlated to RT-qPCR. The authors write about this in the abstract: „*In contrast, three Ag-RDTs demonstrated a more significant correlation with cell culture infectivity (61.8–82.4%)*“. This means that from those samples that were positive in the antigen test, a positive result was also seen in the virus culture with a significantly higher hit rate than with the significantly more sensitive RT-qPCR "postives".

A recently published study by the CDC also points to the high concordance of the antigen test with actual interrogatable virus in a sample from symptomatic patients (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7821766/). Here, a commercial rapid antigen test was matched with a virus cultured in cell culture and RT-qPCR. It showed a high hit rate (positive result) of the antigen test only when the samples also contained replicating viruses. Here, viruses could be grown from 85 of the total 147 samples (=58%) which were positive in the rapid antigen test and RT-qPCR (here with a CT of approx. 22), but only from 11 of the 124 samples (= 9%) which were RT-qPCR positive (here with a CT of 33-34) but antigen rapid test negative.

Another study from the CDC (https://academic.oup.com/cid/advance-article/doi/10.1093/cid/ciab303/6224406) correlated a positive antigen test with a CT of less than 29, and the cultivability of virus in cell culture (also to CT29). All subject samples positive in RT-qPCR with a CT ≥35 were negative in antigen detection.

In a recent publication of the working group of Christian Drosten (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8026170/ he is the last author), in which different antigen tests are tested for reliability, the correlation "positive antigen test" and viral load as well as infectivity is explicitly described. Already in the abstract it says: „*The sensitivity range of most AgPOCTs overlaps with* ***SARS-CoV-2 viral loads typically observed in the first week of symptoms, which marks the infectious period in most patients****. The AgPOCTs with limit of detections that approximate virus concentrations at which patients are infectious might enable shortcuts in decision making in various areas of health care and public health*.” A viral load of 2-9x10^6 copies per smear is specified as the detection limit, which would nevertheless only lead to success (= sufficiently high viral load) in 1/5 of cases when compared with virus cultivation in cell culture. „*In terms of analytical sensitivity, the detection range of most AgPOCTs was found to range between around 2 million and 9 million copies per swab (accounting for a systematic predilution), and thus corresponds to a* *concentration that can be expected to yield a virus isolation success rate of around 20% in cell culture*"

In general, it can be stated from these published data:

- Samples from which viruses can be grown in cell culture, i.e., which have a high (infectious) viral load, are identified with good accuracy by the rapid antigen tests and by RT-PCR with low CT (below 25), but in the vast majority originate from symptomatic individuals.
- Samples from which no viruses can be grown in cell culture are mostly negative in evaluated and correctly applied rapid antigen tests (except for false positives - see 2.3.3) and have high CT values (mostly above 33) in RT-qPCR. These samples are predominantly from asymptomatic tested individuals and prove that these random "positives" without clinical symptoms do not have an infectious viral load.

2.3.3 Reliability (specificity) - exclusion of false positive results

Many of the rapid antigen tests used have not yet undergone a regular conformity assessment procedure for CE marking and have so far only been granted special approval by the BfArM in accordance with §11 of the Medical Devices Act (https://www.bfarm.de/DE/Medizinprodukte/Antigentests/_node.html). In addition, these tests are widely performed by untrained, non-medical personnel or even as "self-tests".

Regarding this problem of performing rapid antigen tests, Professor Oliver Keppler, M.D., chief of virology at the Max Pettenkofer Institute at Munich's Ludwig Maximilian University, calls for the following in an article in the Jan. 13, 2021, issue of Pharmazeutische Zeitung (DOI: 10.1007/s00430-020-00698-8): "[....] *these tests would also absolutely have to be performed correctly. "This should be in the hands of trained professionals*," he says. "*Now there is the idea of recruiting large numbers of job seekers to perform such tests in nursing homes. **If untrained personnel are used, I'm concerned that the reliability of the test results will suffer even further***"

In a recent interview (https://www.br.de/nachrichten/wissen/virologe-keppler-kritisiert-corina-schnelltests-falsche-sicherheit,SUg0dZZ), O. Keppler comments on the question "*How reliable is a positive test result?*" as follows: "*Unfortunately, there are also problems with the specificity of the rapid antigen tests: Depending on the incidence and the test used, according to RKI data**, there are about ten "false" positives for every "true" positive**. This also has serious consequences for the person affected: Immediate report to the health department and quarantine until a negative PCR is obtained, compile contact lists. This causes a great deal of expense, loss of work and school for several days, and last but not least, unwarranted anxiety. It also further undermines confidence in the national testing strategy*."

A recent Cochran review article (https://www.cochrane.de/de/news/aktualisierter-cochrane-review-bewertet-zuverl%C3%A4ssigkeit-von-schnelltests-zum-nachweis-von-covid) also concludes that rapid antigen tests are significantly more reliable in symptomatic individuals than in asymptomatic tested individuals. However, even in symptomatic individuals, the reliability of the best of the rapid tests evaluated in this study is significantly limited, leading the authors to describe the following scenarios:

1. "In a population of 1000 symptomatic individuals, 50 of whom actually have COVID-19, these rapid tests can be expected to correctly identify approximately 40 individuals as COVID-19 infected and miss between 6 and 12 cases of COVID-19. Between 5 and 9 of the positive test results would turn out to be false positives upon review."
2. "In a group of 10,000 persons without symptoms, in which 50 persons are truly infected with SARS-CoV-2, between 24 and 35 persons would be correctly identified as virus carriers, and between 15 and 26 cases would be missed. One would have to expect that the tests would yield between 125 and 213 positive results and that between 90 and 189 of these positive results would actually be false positives.

Also in the current publication of the working group of C. Drosten at the Charité the problem of "false positives" - here however under controlled laboratory conditions - is pointed out and discussed. The tests reviewed reacted falsely positive with various common respiratory viruses -which was confirmed by negative RT-qPCR using E-gene detection. "*All negative samples that showed a SARS-CoV-2 false-positive result in AgPOCTs were retested and* ***confirmed as false-positive*** *with SARS-CoV-2 RT-rtPCR*." (page. 5 in https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8026170/ top.) The authors around C. Drosten consider a false positive rate of up to 3% as acceptable and in two tests even found exceptions of 5% false positives. „*We observed acceptable rates of false-positive results (<3%) with most AgPOCTs, but rates greater than 5% with two assays in particular"*

Thus, even under controlled laboratory conditions with skilled personnel, an average of 30 false positive results would be obtained with commercial rapid tests out of 1000 rapid tests due to cross-reactivity with other respiratory viruses or "unknown factors", according to the publication by C. Drosten. („*thus, a specific factor other than the tested pathogens was likely to have caused positive signals*")

The fact that the high rate of false positive tests in large-scale testing in the population occurs at a time of low viral incidence is shown by an article in the German Ärztezeitung of 04.07.2021 (https://www.aerztezeitung.de/Wirtschaft/80-Prozent-der-positiven-Corona-Schnelltests-falsch-positiv-421053.html). At the end of the regular cold season (May), about 50% of rapid tests were already reported as false positive, and this rate increased until it reached 80% false positive tests in June. The data of the article are based on the information of the Hamburg senate on a small inquiry of the CSU parliamentary group. The evaluation was based on a total of 308,000 reported antigen rapid tests in Hamburg of which 218 tests were positive (= 0.07% of those tested) and after PCR confirmation then only 44 (=0.014% of all those tested) remain.

For the consequences of false positive results due to lack of test specificity, see under 2.3.1. "Pre-test probability

2.5 Conclusion

The rapid antigen tests used for mass testing **cannot provide any information about infectivity**, since they can only detect protein components without any connection to an intact, replicable virus. 1.

- In order to allow an estimation of the infectivity of the tested persons, the respective positive test (similar to RT-qPCR) would have to be individually compared with a cultivability of viruses from the test sample, which is impossible under the extremely variable and unverifiable test conditions.
- The low specificity of the tests causes a high rate of false positives, which result in unnecessary personnel (quarantine) and societal (e.g. schools closed, "outbreak notifications") efforts until they turn out to be false alarms.